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ORIGINAL ARTICLE

JunD is involved in the antiproliferative effect of Δ^9-tetrahydrocannabinol on human breast cancer cells

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It has been recently shown that cannabinoids, the active components of marijuana and their derivatives, inhibit cell cycle progression of human breast cancer cells. Here we studied the mechanism of Δ^9-tetrahydrocannabinol (THC) antiproliferative action in these cells, and show that it involves the modulation of JunD, a member of the AP-1 transcription factor family. THC activates JunD both by upregulating gene expression and by translocating the protein to the nuclear compartment, and these events are accompanied by a decrease in cell proliferation. Of interest, neither JunD activation nor proliferation inhibition was observed in human non-tumour mammary epithelial cells exposed to THC. We confirmed the importance of JunD in THC action by RNA interference and genetic ablation. Thus, in both JunD-silenced human breast cancer cells and JunD knockout mice-derived immortalized fibroblasts, the antiproliferative effect exerted by THC was significantly diminished. Gene array and siRNA experiments support that the cyclin-dependent kinase inhibitor p27 and the tumour suppressor gene testin are candidate JunD targets in cannabinoid action. In addition, our data suggest that the stress-regulated protein p8 participates in THC antiproliferative action in a JunD-independent manner. In summary, this is the first report showing not only that cannabinoids regulate JunD but, more generally, that JunD activation reduces the proliferation of cancer cells, which points to a new target to inhibit breast cancer progression.
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Keywords: cannabinoids; breast cancer; JunD; AP-1; p27; testin

Introduction

There is increasing evidence that cannabinoids, the active components of marijuana and their derivatives, possess antitumoural properties. Thus, a wide variety of cannabinoid compounds, including Δ^9-tetrahydrocannabinol (THC, the most abundant and potent plant-derived cannabinoid) and other phytocannabinoids (cannabinol, cannabidiol), endocannabinoids (endogenously produced cannabinoids) and synthetic cannabinoids, exerts antiproliferative actions on a wide spectrum of tumour cells *in vitro* (Guzman, 2003). This effect has been confirmed in animal models of lung, pancreas, skin and breast carcinomas, glioma, thyroid epithelioma, lymphoma and melanoma (Guzman, 2003; Blazquez *et al*., 2006; Carracedo *et al*., 2006a; Ligresti *et al*., 2006). Cannabinoids exert most of their antiproliferative actions through activation of specific G-protein-coupled receptors. So far, two cannabinoid receptors—CB_1 and CB_2—have been cloned and characterized from mammalian tissues (Guzman, 2003). They differ mainly in their tissue-expression pattern: although CB_1 is mostly present in the brain, peripheral nerve terminals and other non-neural sites such as testis, eye, vascular endothelium and spleen, CB_2 expression is almost restricted to the immune system (Guzman, 2003). Engagement of these receptors modulates signalling pathways critically involved in the control of cell growth and survival (Guzman, 2003). It has been proposed that cannabinoids exert their antiproliferative effects on human breast cancer cells (HBCCs), at least in part, by controlling the progression through the cell cycle (De Petrocellis *et al*., 1998; Caffarel *et al*., 2006; Sarnataro *et al*., 2006). In particular, THC induces cell-cycle arrest at the G_2–M transition by downregulating cyclin-dependent kinase 1 (CDK1, Cdc2) (Caffarel *et al*., 2006). However, the molecular bases of this cannabinoid effect are as yet unknown. Here we therefore investigated the mechanism underlying cannabinoid antiproliferative action in HBCCs.

Results

THC upregulates JunD levels in breast cancer cells but not in non-tumour breast cells

We have previously reported that THC reduces HBCC proliferation by blocking the progression through the

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cell cycle (Caffarel *et al*., 2006). To study the mechanism of cannabinoid action, we performed a DNA microarray-based analysis of HBCC response to THC. Specifically, we evaluated the gene-expression profile of EVSA-T cells treated with THC (3 or 5 μM) for 8 or 24 h. The data obtained for each condition are shown in Supplementary Tables 3–7. A hierarchical unsupervised analysis of microarray data pointed to the existence of three different populations corresponding to vehicle-, 3 and 5 μM THC-treated cells, suggesting that the cannabinoid dose-dependently modulates the expression of a number of genes (Figures 1a and b). Irrespective of the dose and the time of treatment, THC seemed to impact biological functions such as metabolism, transcription and cell cycle (Figure 1c and Supplementary Table 3). We further analysed the gene-expression profile of EVSA-T cells treated with 5 μM THC (the dose that evokes the antiproliferative effects previously described (Caffarel *et al*., 2006)) for 8 h (a time at which no significant changes in cell proliferation are evident yet (Caffarel *et al*., 2006)). A group of 28 discriminatory genes was obtained in THC-treated cells, 12 of which were downregulated and 16 of which were upregulated (Supplementary Table 4). The expression of these genes, except for JTV1, was not modified when cells were exposed to the lower THC concentration (3 μM, Supplementary Table 5), suggesting that these particular genes may be involved in the antiproliferative effect induced by 5 μM THC.

Assuming that the mechanism underlying THC effect should involve an initial modulation of the expression of different genes, we paid a special attention to the transcription control-related THC-modulated genes. We found three genes involved in transcription: Id2, JunD and Zfp36L1. Because of the importance of the activator protein-1 (AP-1) family in biological processes related to cell proliferation, survival and death, we decided to focus on JunD. JunD is a member of the AP-1 family transcription factor, which consists of dimeric complexes formed by Jun, Fos or ATF members (Mechta-Grigoriou *et al*., 2001; Eferl and Wagner, 2003). These complexes are involved in a wide range of transcriptional regulatory processes linked to cellular proliferation, differentiation and survival (Mechta-Grigoriou *et al*., 2001; Eferl and Wagner, 2003). By means of PCR, we confirmed that JunD mRNA levels were upregulated upon cannabinoid challenge (Figure 2a). Interestingly, THC did not modify JunD mRNA in non-transformed human mammary epithelial cells (HMECs) (Figure 2b), which are resistant to THC growth-inhibitory action (Caffarel *et al*., 2006). The expression of other AP-1 family members was also analysed. At early time points (compatible with the kinetics of AP-1 activation that have been previously reported in various cell types using different stimuli (Lallemand *et al*., 1997)), we found an increase in c-Jun, c-Fos, FosB and Fra-2 mRNAs, together with a decrease in Fra-1 mRNA (Figure 2c). We also confirmed by PCR the upregulation observed in the microarrays (Supplementary Table 4) of Cdkn1B (p27) (a CDK inhibitor family member that blocks the entrance into the S phase of the cell cycle (Malumbres and Barbacid, 2001)), and testin (Tes) (a cytoskeleton-associated protein that has been recently identified as a tumour suppressor gene (Drusco *et al*., 2005)) (Figure 2a). No changes were observed in the expression of these two genes in HMECs (Figure 2b). Finally, we validated the downregulation by THC of Sfrs3 (SRP20) and Sfrs7 (9G8) (Figure 2a), two members of the arginine/serine-rich splicing factor family whose altered expression and improper pre-mRNA processing have been associated to tumourigenesis (Stickeler *et al*., 1999).

THC activates JunD by upregulation of protein levels and translocation to the nucleus

We subsequently analysed whether the observed changes in JunD mRNA translated into changes in JunD protein levels, intracellular distribution and/or transcriptional activity. THC increased JunD protein levels (Figure 3a). Surprisingly, although it is well established that the AP-1 family members are solely located in the nucleus (Pfarr *et al*., 1994; Mechta *et al*., 1997; Eferl and Wagner, 2003), JunD protein was also detected in the cytoplasm of EVSA-T cells (Figures 3b–d). Moreover, THC seemed to induce the intracellular relocation of JunD in our system. Thus, cannabinoid challenge increased JunD nuclear staining (Figures 3b and c). Both the decrease in cytoplasmic JunD and the increase in nuclear JunD upon THC treatment were confirmed by western blot (Figure 3d). Since no JunD-specific target genes have been described so far, we analysed the effect of THC on AP-1 transcriptional activity. EVSA-T cells were thus transfected with a c-Jun promoter (which contains an AP-1 site)-luciferase construct or the corresponding one mutated in the AP-1-binding site. THC increased luciferase activity in c-Jun-luc-transfected cells (Figure 4a) and this effect was efficiently abolished when the AP-1 site was truncated (Figure 4a) or when JunD was silenced (Figure 4b), supporting that THC increases AP-1 transcriptional activity through JunD. We are aware that the intensity of the transcriptional activation was modest, most likely owing to the high basal activity of the c-Jun promoter detected in our system, as indicated by the much lower levels of luciferase activity detected in ΔAP-1-luciferase-transfected cells compared to intact AP-1-luciferase partners (Figure 4a). The constructions used in this study contain the proximal AP-1 site in the c-Jun promoter, which includes a TPA responsive element-like sequence preferentially recognized by c-Jun/ATF-2 heterodimers rather than conventional c-Jun/c-Fos dimers (van Dam *et al*., 1993). To further demonstrate that JunD is a significant activator of the AP-1 site in the c-Jun promoter, we co-transfected EVSA-T cells with c-Jun-luc and JunD/c-Fos or c-Jun/Fra-1 chimaeras and observed that they induced a very similar transcriptional activation compared to that induced by THC (JunD/c-Fos: 1.6 times vs control cells; c-Jun/Fra-1: 2.2 times vs control cells). These data support that in the present cellular context (1) JunD-containing dimers transactivate the c-Jun promoter and (2) THC- and JunD-induced

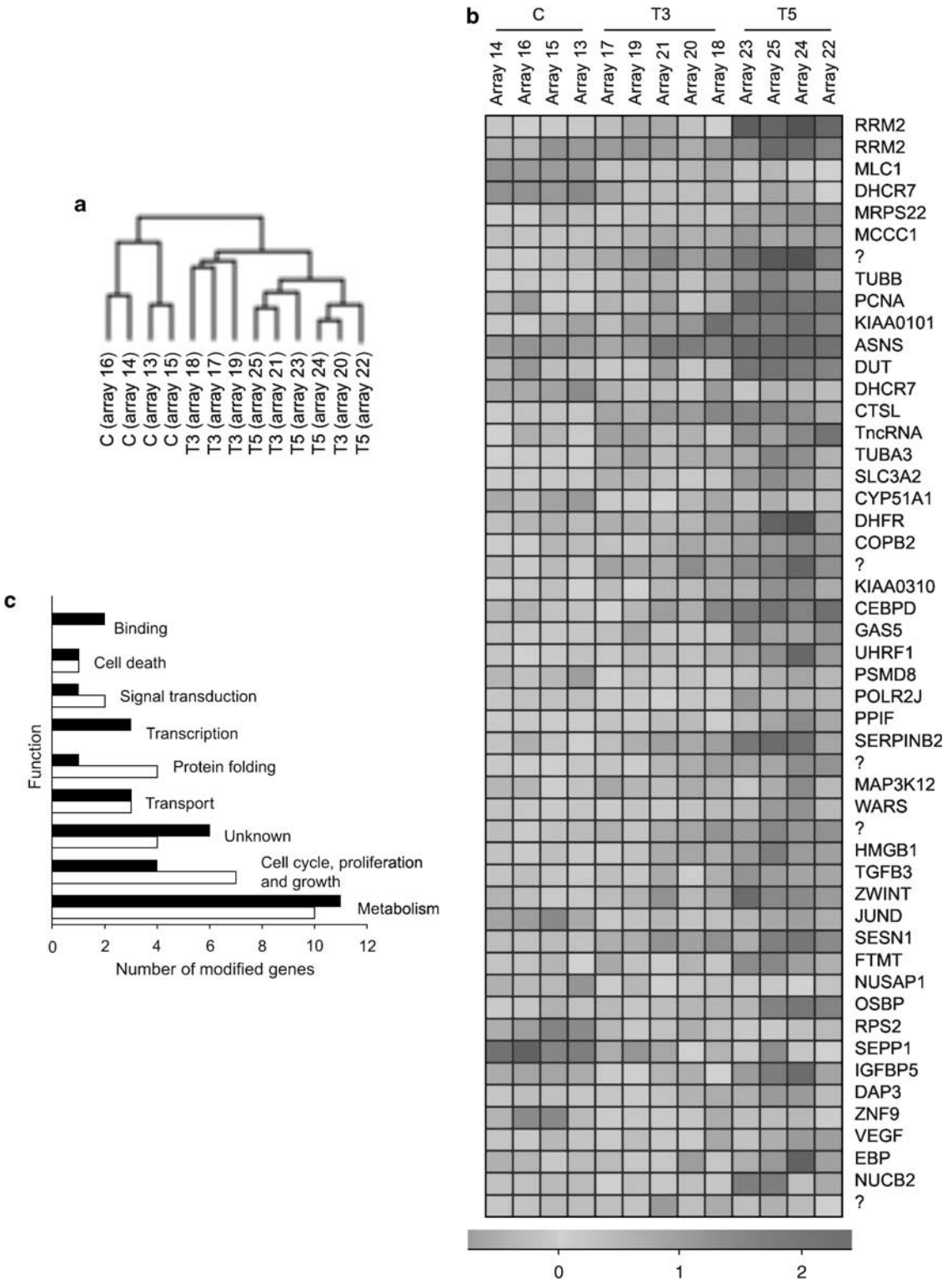

Figure 1 Analysis of microarray data obtained from EVSA-T cells treated with vehicle (C) or Δ^9-tetrahydrocannabinol (THC, 3 or 5 μM, T3 and 5, respectively) for 24 h. (**a**) Conditions were classified by a hierarchical unsupervised method. The tree represents Euclidean (normal) distances. (**b**) Cluster analysis of the different arrays (see Supplementary Table 1) distributed in three categories: vehicle-, 3 and 5 μM THC-treated cells. (**c**) Comparison of the gene-expression patterns of THC-treated (3 and 5 μM, 8 and 24 h) vs vehicle-treated cells. Bars represent the number of downregulated (open bars) and upregulated (closed bars) genes.

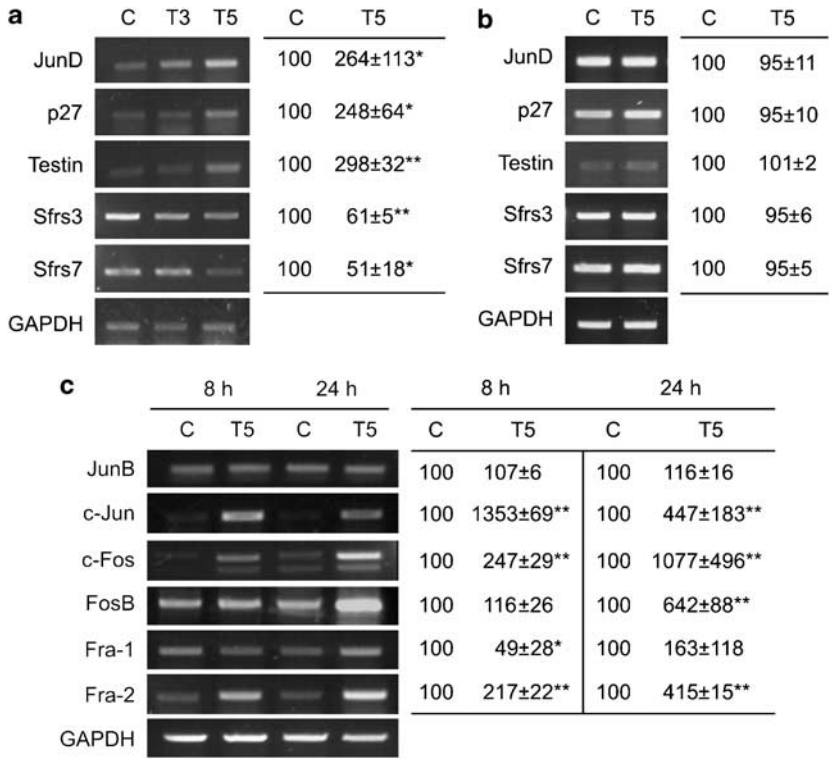

Figure 2 Validation of microarray data by reverse transcription (RT)–PCR and real-time quantitative (RT-q)PCR. The expression of certain genes selected from the microarrays was analysed after 8 h of Δ^9-tetrahydrocannabinol (THC) challenge in EVSA-T cells (**a**) and human mammary epithelial cells (HMECs) (**b**). (**c**) Analysis of activator protein-1 (AP-1) family members in EVSA-T cells. Representative images of RT–PCRs ($n=4$) are shown in each panel. Values correspond to relative expression vs vehicle-treated cells as determined by RT-qPCR (set at 100%; $n=4$; $^*P<0.05$; $^{**}P<0.01$). C, control; T3 and T5, THC 3 and 5 μM, respectively.

c-Jun promoter transactivation is as efficient as that produced by c-Jun, which is a preferred activator of this particular AP-1 site (van Dam *et al*., 1993).

JunD is involved in THC antiproliferative effect in HBCCs

To test the involvement of JunD in THC antiproliferative action, we selectively knocked down its mRNA by RNA interference. As shown in Figure 5a, JunD-silenced EVSA-T cells were more resistant to THC than the corresponding control small-interfering RNA (siRNA)-transfected cells. Selective JunD knockdown was verified by PCR and western blot (Figure 5a, inset). In addition, the decrease in Cdc2 levels associated to cannabinoid-evoked cell cycle arrest described previously (Caffarel *et al*., 2006) was confirmed in the microarrays (Supplementary Table 7) and abolished upon JunD silencing (Figure 5b). In order to evaluate the selectivity of JunD in THC signalling, we determined whether the observed increase in c-Jun (Figure 2c) was also involved in THC antiproliferative action. For that purpose, we knocked down its mRNA with three different specific siRNAs and analysed cell viability upon cannabinoid challenge. No significant changes were observed between control and c-Jun siRNA-transfected cells (Figure 5c). We next studied the possible involvement of Tes and p27 in THC-induced JunD-mediated antiproliferative effect. Thus, THC-induced changes in Tes and p27 mRNA were attenuated in JunD-silenced cells (Figure 6a). Of interest, c-Jun silencing did not significantly modify the induction of p27 and Tes elicited by THC (data not shown), suggesting that these genes are largely under the control of JunD. As expected, selective p27 or Tes knockdown with specific siRNAs diminished the antiproliferative effect of THC (Figure 6b, left panel). Silencing of p27 did not prevent the increase in JunD and Tes mRNA levels elicited by THC. In contrast, Tes silencing completely blocked THC-induced JunD and p27 upregulation (Figure 6b, right panel). All these findings indicate that Tes and JunD are functionally interrelated, and that they two are upstream of p27.

It has been recently shown that the stress-regulated protein p8 is an essential mediator of cannabinoid antitumoral action in gliomas and pancreatic adenocarcinomas (Carracedo *et al*., 2006a, b). To investigate whether this protein is also involved in THC-induced antiproliferative effect in HBCCs, we first analysed its expression upon cannabinoid challenge. As shown in Figure 6c, THC induced a dose-dependent increase in p8 levels in transformed cells, whereas no significant

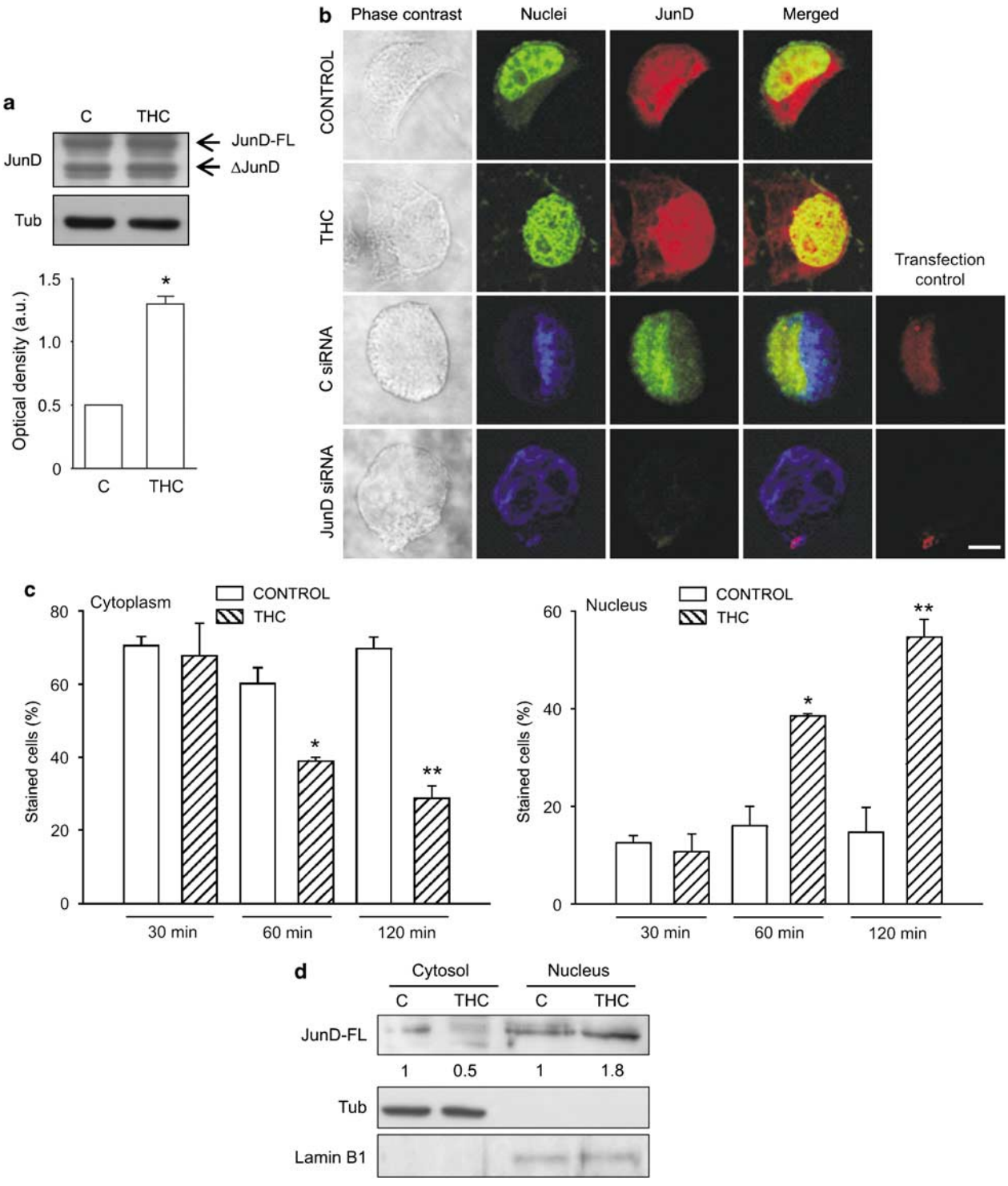

Figure 3 Analysis of JunD protein expression and intracellular localization in EVSA-T cells. (**a**) Western blot of JunD after 30 min exposure to 5 μM Δ^9-tetrahydrocannabinol (THC). Arrows point to the two isoforms of JunD: the full-length isoform (JunD-FL) and the truncated isoform (ΔJunD). The graph represents the densitometric analysis of protein levels. Results are expressed as optical density (arbitrary units) relative to control cells set at 1. (**b**, **c**) Confocal microscopy analysis of JunD intracellular localization. Upper two rows in **b**, cells were challenged with 5 μM THC for 120 min. JunD and cell nuclei were stained in red and blue, respectively. For visualizing purposes, nuclear blue signal has been turned green. Lower two rows in **b**, cells were transfected with control or JunD small-interfering RNA (siRNA) and JunD was stained in green. A fluorescent-labelled siRNA (red signal) was used as transfection marker. Representative images are shown. Scale bar: 9 μm. (**c**) The percentage of cells expressing JunD mostly in the cytoplasm or mostly in the nucleus was determined. One hundred cells per slide and three different slides per condition were counted. (**d**) Western blot of JunD in cytosolic and nuclear protein extracts after 120 min exposure to 5 μM THC. α-Tubulin and lamin B1 were used as markers of cytosolic and nuclear fractions, respectively. Numbers represent optical density (arbitrary units) relative to control cells set at 1. Representative experiments are shown in **a**, **b** and **d** ($n=3$). *$P<0.05$; **$P<0.01$; vs control cells.

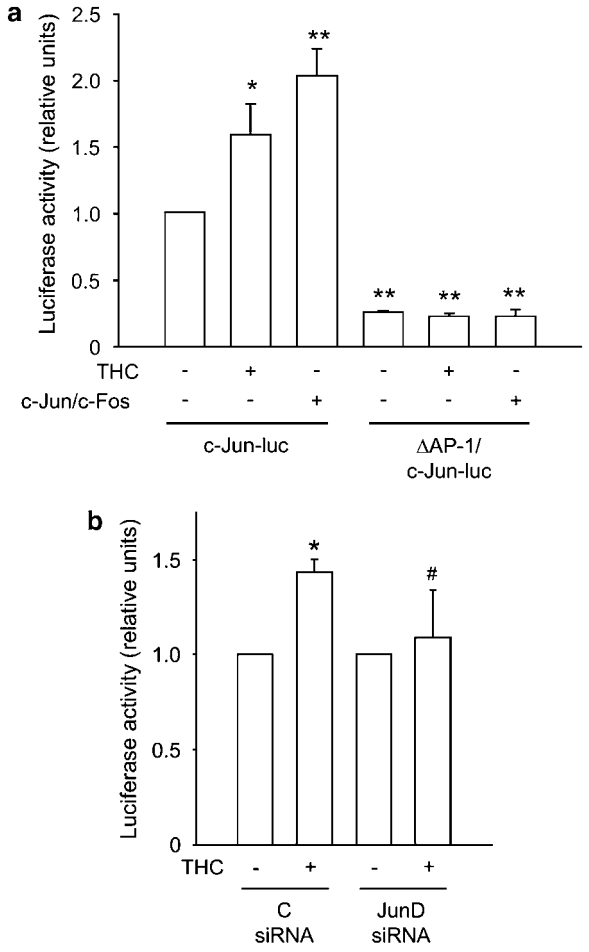

Figure 4 Analysis of activator protein-1 (AP-1) transcriptional activity. (**a**) EVSA-T cells were transfected either with a c-Jun promoter-luciferase construct (c-Jun-luc) or with a similar construct with the AP-1 site located in the c-Jun promoter mutated (ΔAP-1/c-Jun-luc). Luciferase activity was determined 8 h after Δ^9-tetrahydrocannabinol (THC, 5 μM) challenge. A c-Jun/c-Fos chimaeric plasmid was used as positive control of AP-1 transcriptional activation. (**b**) Cells were transfected with control or JunD small-interfering RNA (siRNA) and 24 h later with the c-Jun-luc plasmid, and challenged as above. A renilla control reporter vector was used for normalization. The graphs represent luciferase activity (expressed as luciferase signal/renilla signal) relative to the control (c-Jun-luc-transfected vehicle-treated cells) set at 1 ($n=3$). $^*P<0.05$; $^{**}P<0.01$ vs control cells; $^{\#}P<0.05$ vs C siRNA-transfected THC-treated cells.

changes were detected in non-transformed HMECs. Moreover, p8 silencing partially prevented the antiproliferative effect of THC (Figure 6d, left panel). However, in our system, p8 seems to act in a JunD-independent manner as its selective knockdown did not significantly affect the upregulation of JunD, p27 or Tes elicited by THC (Figure 6d, right panel), and JunD silencing did not affect THC-induced p8 upregulation (relative p8 expression vs the corresponding vehicle-treated cells set at 1: control siRNA-transfected THC-treated cells: 1.9 ± 0.5; JunD siRNA-transfected THC-treated cells: 1.9 ± 0.2).

JunD genetic ablation confers resistance to THC antiproliferative effect

To further verify the involvement of JunD in THC action, we compared the effect of THC on wild-type vs $JunD^{-/-}$ immortalized fibroblasts. As for JunD siRNA-knocked-down EVSA-T cells, fibroblasts lacking JunD expression were more resistant to THC than their wild-type counterparts (Figure 7a). The presence of CB_1 and CB_2 cannabinoid receptors was evidenced in both wild-type and $JunD^{-/-}$ immortalized fibroblasts (Figure 7b). Moreover, the antiproliferative effect of THC on wild-type cells was attenuated by a combination of CB_1 and CB_2 selective antagonists (Figure 7c), suggesting that these receptors are not only present but also active in these cells. It has been recently proposed that JunD protects cells from oxidative stress (Gerald *et al*., 2004). In addition, THC and other structurally related cannabinoids have intrinsic antioxidant properties (Hampson *et al*., 1998, 2000) and in certain physiopathological contexts they have the ability to reduce cellular reactive oxygen species (ROS) content (Iuvone *et al*., 2004). To analyse whether the increased resistance to THC shown by JunD-deficient cells was due to a THC-induced decrease in ROS generation, we measured ROS content in wild-type and JunD-deficient fibroblasts. As shown in Figure 7d, JunD-deficient cells had higher basal ROS levels, but no decrease in ROS content was observed in these cells upon cannabinoid challenge.

Discussion

Cannabinoids possess antineoplasic properties in a wide plethora of animal models of cancer (Guzman, 2003; Blazquez *et al*., 2006; Carracedo *et al*., 2006a; Ligresti *et al*., 2006). The only related clinical trial performed to date points to a decreased proliferation of tumour cells in THC-treated recurrent glioblastoma patients, and to an overall outcome, in terms of median survival, similar to that obtained with temozolomide, the current benchmark for the management of malignant gliomas (Guzman *et al*., 2006). The mechanisms underlying cannabinoid antitumoural action include antiproliferative, proapoptotic and antiangiogenic effects (Guzman, 2003). Data presented herein demonstrate the involvement of the AP-1 transcription factor JunD in the antiproliferative effect of THC in breast cancer cells. Nonetheless, considering the intricacy of AP-1 complex regulation, which includes regulation of phosphorylation states as well as protein–protein and protein–DNA interactions (Mechta-Grigoriou *et al*., 2001; Eferl and Wagner, 2003), the participation of other AP-1 family members in THC signalling may not be ruled out. Moreover, we cannot discard the participation of other THC-modulated transcription factors such as Id2 and Zfp36L1, or even other non-transcription-related genes, in the primary events underlying the antiproliferative effect described herein. We also show that THC induces a JunD-mediated upregulation of p27 and Tes, although further analysis of the regulatory features of these two

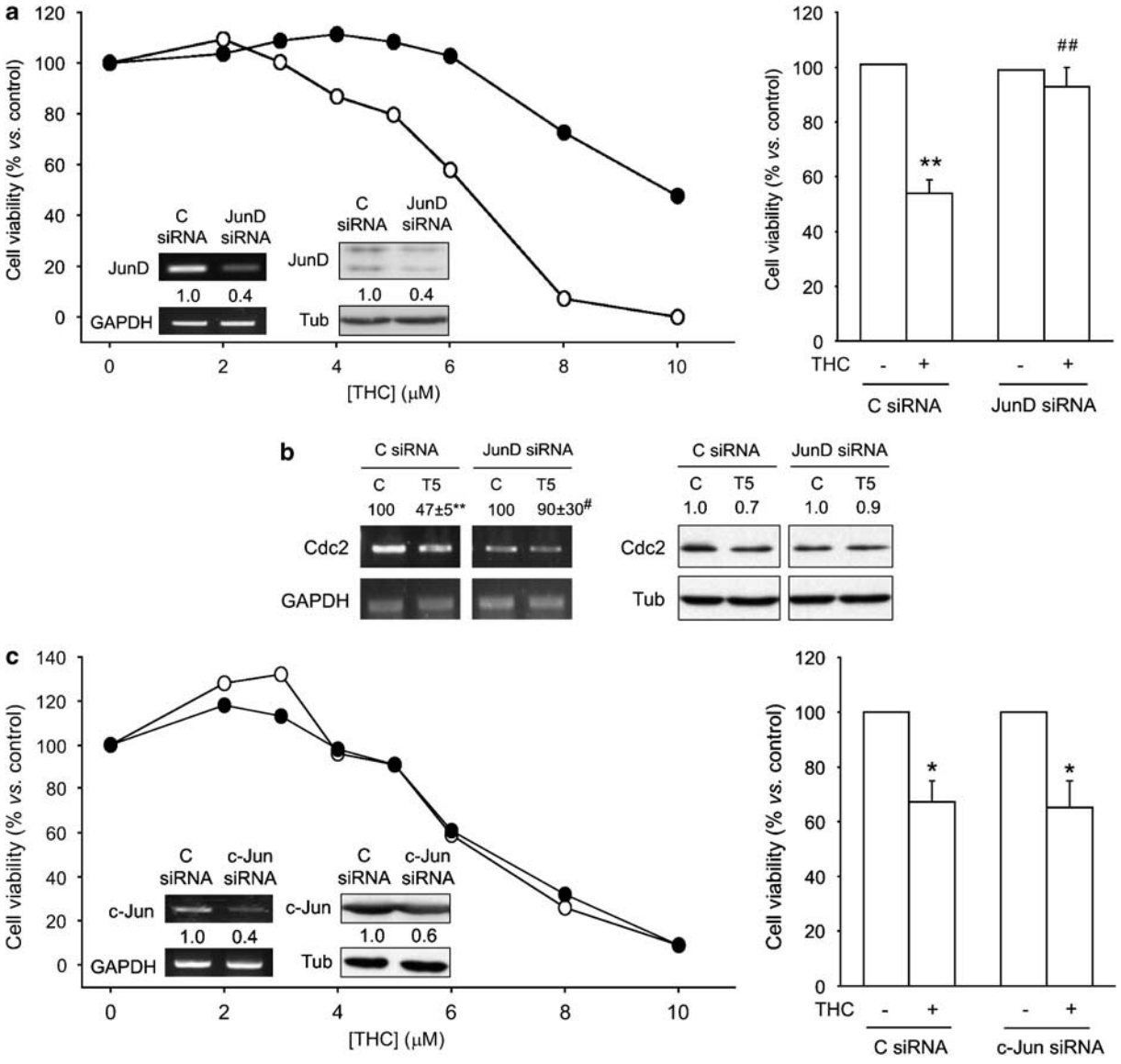

Figure 5 JunD knockdown in EVSA-T cells. (**a**, **c**) Left panels, cells were transfected with JunD small-interfering RNA (siRNA) (**a**, black circles), c-Jun siRNA (**c**, black circles) or control siRNA (**a** and **c**, white circles) and cell viability was determined 24 h after Δ^9-tetrahydrocannabinol (THC) challenge. Representative experiments ($n=4$) are shown. Insets, JunD (**a**) and c-Jun (**c**) expression as analysed by reverse transcription (RT)–PCR (left, images), real-time quantitative (RT-q)PCR (left, numbers) and western blot (right), 48 h after siRNA transfection. Values represent mRNA (left) and protein (right) levels relative to control siRNA-transfected cells set at 1. Representative experiments are shown ($n=3$). Right panels, cells were transfected with three different JunD siRNAs (**a**), three different c-Jun siRNAs (**c**) or with control siRNA (**a**, **c**) and exposed to 6 μM THC for 24 h. Two different experiments were performed with each JunD or c-Jun siRNA (total $n=6$ for each gene). Values represent viable cells vs the corresponding vehicle-treated cells set at 100%. (**b**) Expression of Cdc2 as analysed by RT–PCR (left panel, images), RT-qPCR (left panel, numbers) and western blot (right panel). JunD or control siRNA-transfected cells were exposed to 5 μM THC (T5) or vehicle (C) for 8 h. Values represent relative mRNA (left) and protein (right) levels vs control cells set at 100% and 1, respectively ($n=3$). $^{*}P<0.05$; $^{**}P<0.01$ vs control cells; $^{\#}P<0.05$; $^{\#\#}P<0.01$ vs C siRNA-transfected THC-treated cells.

genes is required to determine whether they are directly modulated by JunD-containing dimers. A JunD-independent involvement of the stress-regulated protein p8 has also been found. Interestingly, the expression of either JunD, p27, Tes or p8 is not affected by THC in non-tumour mammary epithelial cells, which had been previously reported as resistant to THC antiproliferative action (Caffarel *et al*., 2006). It has been widely shown that cannabinoids exert antiproliferative actions on cultured tumour cells without affecting the proliferation of their non-transformed counterparts. Thus, although several astrocytoma, neuroblastoma, thyroid epithelioma and skin carcinoma cells are killed by cannabinoids, these compounds do not affect the viability of primary astrocytes, neurons, thyroid epithelial cells or keratinocytes in culture (reviewed in Guzman (2003). The same observation was reported later for HBCCs and a non-transformed epithelial counterpart (Caffarel *et al*., 2006)

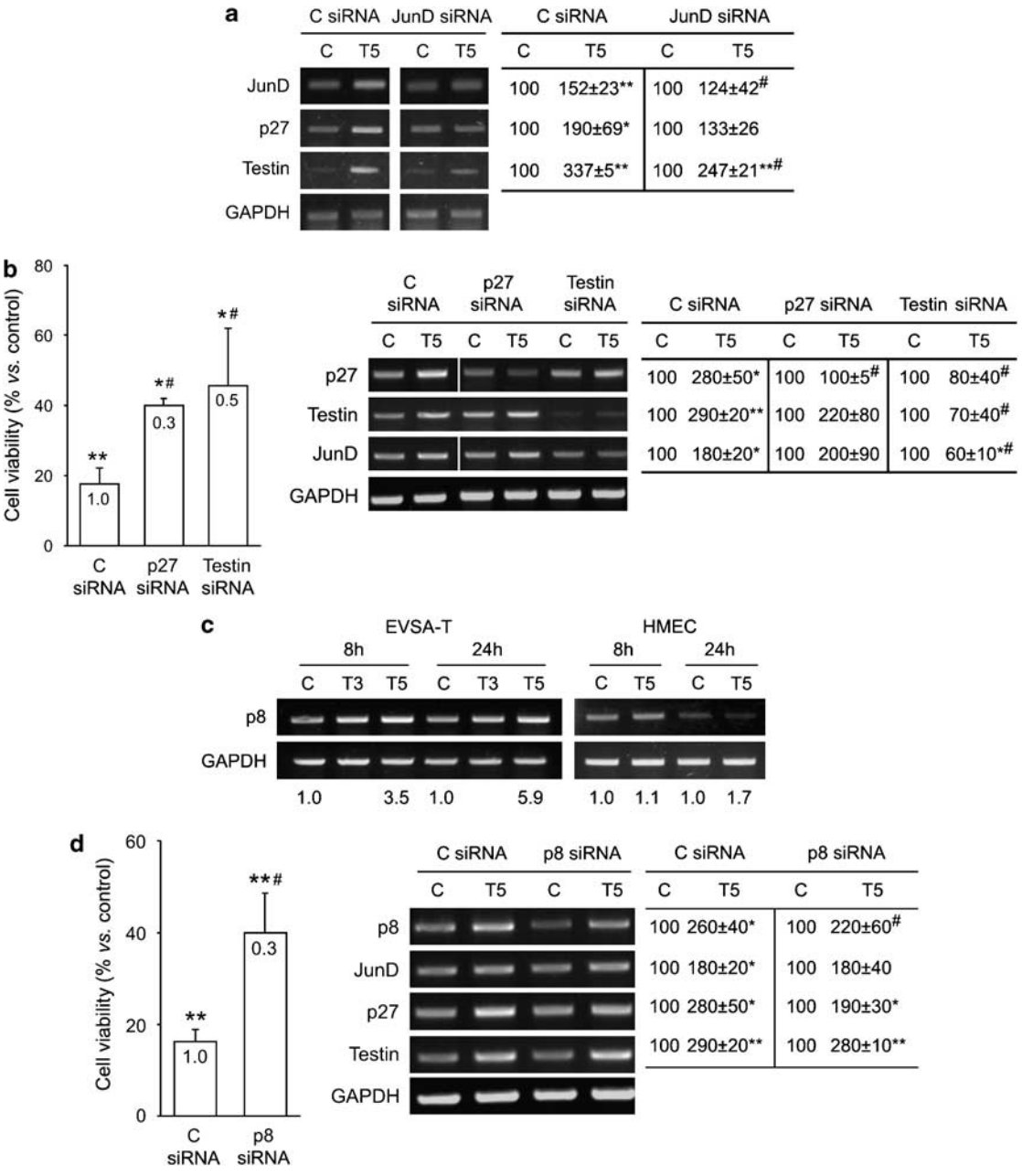

Figure 6 Analysis of the involvement of p27, testin and p8 in Δ^9-tetrahydrocannabinol (THC) action. (**a**) Expression of p27 and testin as analysed by reverse transcription (RT)–PCR (images) and real-time quantitative (RT-q)PCR (table). JunD or control small-interfering RNA (siRNA)-transfected cells were exposed to 5 μM THC (T5) or vehicle (C) for 8 h. Values represent relative expression compared to the corresponding vehicle-treated cells set at 100. (**b**) Cells were transfected with the indicated siRNAs. Histogram, relative cell viability of THC-treated cells vs their corresponding siRNA-transfected vehicle-treated cells (set at 100%) 24 h after 8 μM THC challenge. Values within the bars represent the relative expression of p27 (middle bar) and testin (right bar) after selective silencing vs their expression in control siRNA-transfected cells (set at 1) as determined by RTq-PCR. Expression of p27, testin and JunD by RT–PCR (images) and RTq-PCR (table) in cells transfected with the indicated siRNAs and challenged with 5 μM THC for 8 h. Values represent relative expression vs the corresponding vehicle-treated cells set at 100. (**c**) Cells were treated with vehicle (C) or THC (3 or 5 μM, T3 and T5, respectively) for 8 or 24 h and mRNA expression was analysed by RT–PCR. Values correspond to relative expression as determined by RTq-PCR vs control cells set at 1. (**d**) Cells were transfected with p8 siRNA and cell viability (histogram) and expression of the indicated genes (images and table) determined 24 and 8 h after cannabinoid challenge, respectively. Bars represent relative cell viability of THC-treated cells vs their corresponding siRNA-transfected vehicle-treated cells (set at 100%). Values within the bars represent the relative expression of p8 (right bar) after selective silencing vs its expression in control siRNA-transfected cells (set at 1) as determined by RTq-PCR. Values in the table correspond to relative expression vs the corresponding vehicle-treated cells set at 100. $^*P<0.05$; $^{**}P<0.01$ vs vehicle-treated cells; $^{\#}P<0.05$ vs C siRNA-transfected THC-treated cells.

as well as for melanoma cells and non-transformed melanocytes (Blazquez *et al*., 2006). The reason for the different sensitivity to THC of tumour cells and non-transformed cells is currently unknown. The hypothesis that this difference might be due to distinct expression patterns of cannabinoid receptors is however unlikely.

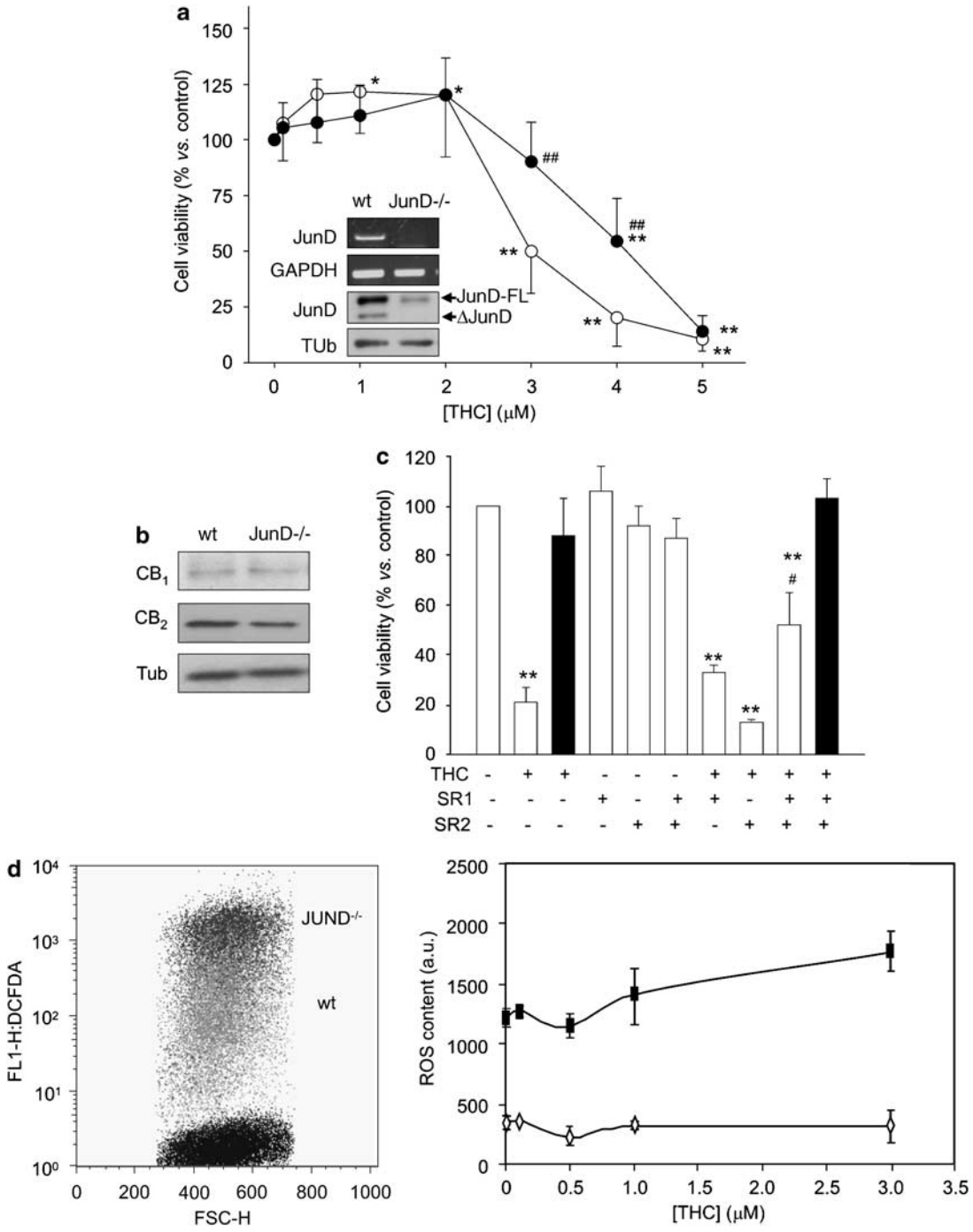

Figure 7 JunD knockout in mouse immortalized fibroblasts. (**a**) Wild-type (white circles) and JunD-deficient cells (black circles) were incubated with Δ^9-tetrahydrocannabinol (THC) for 72 h. Values represent viability of THC-treated cells ($n=3$) relative to that of vehicle-treated cells set at 100 for each THC concentration ($^{*}P<0.05$; $^{**}P<0.01$ vs control cells; $^{\#\#}P<0.01$ vs THC-treated wild-type cells). Inset, JunD expression as analysed by reverse transcription (RT)–PCR (top) and western blot (bottom). Arrows point to the full-length (JunD-FL) and the truncated (ΔJunD) isoforms of JunD. Representative experiments are shown ($n=3$). (**b**) Western blot of CB_1 and CB_2 receptors in wild-type and JunD-deficient cells. A representative experiment ($n=3$) is shown. (**c**) Viability of wild-type (open bars) and JunD-deficient cells (closed bars) treated with THC (3 μM), selective CB_1 (SR141716 (SR1), 0.1 μM) or CB_2 (SR144528 (SR2), 0.1 μM) receptor antagonists or combinations of the drugs for 72 h. Values represent percentage of viability relative to vehicle-treated cells ($^{**}P<0.01$ vs control cells; $^{\#}P<0.05$ vs cells treated only with THC). (**d**) Relative reactive oxygen species (ROS) content (arbitrary units) in wild-type and JunD-deficient cells as determined by CM-H_2DCFDA fluorescence. Left panel, representative fluorescence-activated cell sorting (FACS) analysis of basal levels. Right panel, intracellular ROS content ($n=3$) after THC incubation for 24 h. Black symbols, $JunD^{-/-}$ cells; white symbols, JunD wild-type cells.

Thus, we have found similar levels of CB_1 receptors (hardly detectable) and CB_2 receptors (easily detectable) in EVSA-T cells and HMECs (data not shown). This is in agreement with previous reports showing similar levels of those receptors in THC-sensitive and THC-resistant glioma cell lines (reviewed in Guzman (2003). Likewise, the basal levels of JunD and its targets, p27 and Tes, were not significantly different in EVSA-T cells and HMECs (data not shown). Additional factors must be therefore responsible for the differential effect of THC on breast tumour vs non-tumour cells.

AP-1 family members have been typically associated to cell proliferation, oncogenic transformation, metastasis and angiogenesis (Mechta-Grigoriou *et al*., 2001; Eferl and Wagner, 2003). However, recent studies have revealed that their functions depend on the cellular context, the nature of the stimulus and the particular AP-1 member considered. Specifically, and in line with the present study, JunD overexpression in immortalized fibroblasts inhibits whereas JunD deficiency stimulates cell proliferation (Pfarr *et al*., 1994; Weitzman *et al*., 2000). Moreover, ectopic JunD expression in those cells inhibits oncogenic transformation by activated Ras and reduces tumour angiogenesis by protecting cells from oxidative stress (Mechta *et al*., 1997; Gerald *et al*., 2004).

Previous evidences had indicated that cannabinoids regulate the AP-1 complex in different cellular contexts. For instance, the endocannabinoid 2-arachidonoylglycerol induces differentiation of JB6 epithelial cells through increased AP-1-dependent DNA binding and transcriptional activity (Berdyshev *et al*., 2001; Zhao *et al*., 2005). Of interest, the magnitude of the increase in transcriptional activity described in that report (two to threefold) is very similar to that shown in the present study. In addition, the endocannabinoid anandamide inhibits human keratinocyte differentiation by decreasing AP-1 transcriptional activity (Maccarrone *et al*., 2003) and induces apoptosis of human non-tumour liver cells through overexpression of c-Jun and JunB and stimulation of AP-1 DNA binding (Giuliano *et al*., 2006). However, no such effect was observed in HepG2 hepatoma cells (Giuliano *et al*., 2006). In summary, this is the first report showing not only that the AP-1 complex, particularly JunD, plays a pivotal role in cannabinoid antiproliferative action but, generally speaking, that JunD activation reduces the proliferation of cancer cells. These findings point therefore to a new target to inhibit breast cancer progression, which may contribute to the design of efficient treatments for this malignancy.

Materials and methods

Cell culture and viability

EVSA-T cells, kindly given by Dr Lopez-Rivas (CABD, CSIC, Seville, Spain), were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Immortalized fibroblast lines from wild-type and $junD^{-/-}$ mouse embryos were generated as described (Gerald *et al*., 2004) and maintained in DMEM supplemented with 7% FBS. Cells were transferred to a low (0.5%)-FBS medium immediately before cannabinoid challenge. HMECs, kindly given by Dr Lacal (IIB, CSIC, Madrid, Spain), were grown in mammary epithelial growth medium (Cambrex, East Rutherford, NJ, USA) according to manufacturer's instructions. Cannabinoid compounds were prepared in dimethyl sulphoxide. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (Sigma, St Louis, MO, USA).

Analysis of gene expression by cDNA microarrays

Total RNA was isolated from cells treated with vehicle, 3 or 5 μM THC for 8 or 24 h, as well as from non-treated cells at the time of drug addition ($t=0$, reference), with RNeasy Mini Kit (Qiagen, Hilden, Germany). Double-stranded cDNA was amplified with Superscript Choice System and T7-(deoxythymidine)24 oligo-primers (Life Technologies Inc., Gaithersburg, MD, USA) and in-vitro transcription was carried out with Megascript T7 (Ambion, Austin, TX, USA). Samples were labelled with Cy3-dUTP and Cy5-dUTP (Amersham, Uppsala, Sweden) and hybridized on an advanced version of the Spanish National Cancer Research Centre (Centro Nacional de Investigaciones Oncológicas) Oncochip (http://bioinfo.cnio.es/data/oncochip) as described in the Minimum Information About a Microarray Experiment (MIAME) (Supplementary Table 1). Slides were scanned in a Scanarray 5000 XL scanner (GSI Lumonics Kanata, Ontario, Canada) and the data matrices were processed with GenePix 6.0 software (Axon, Instruments Inc., Union City, CA, USA). Genes with FDR <0.2 and more than twofold changed were considered as significantly modulated. The self-organising tree algorithm program (http://gepas.bioinfo.cnio.es/cgi-bin/sotarray) was used for clustering analysis.

Reverse-transcription and real-time quantitative PCR

RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), including a DNase digestion step, with the Real Star Kit (Durviz, Valencia, Spain), and cDNA was obtained with Transcriptor Reverse Transcriptase (Roche Applied Science, Penzberg, Germany). The primers used for amplification are shown in Supplementary Table 2. For RT-qPCR, probes were from the Universal Probe Library (Roche Applied Science) and 18S RNA was used as reference.

Western blot analysis

Whole cell lysates and cytosolic and nuclear extracts were prepared as described in Carracedo *et al*. (2006a) and Salazar *et al*. (2006), respectively. Blots were incubated with anti-JunD (Pfarr *et al*., 1994), anti-CB_1 (Affinity Bioreagents, Golden, CO, USA), anti-CB_2 (Affinity Bioreagents), anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-lamin B1 (Santa Cruz Biotechnology), anti-Cdc2 (Santa Cruz Biotechnology) or anti-α-tubulin (Sigma) antibodies, which recognize samples from human and murine origins. Luminograms were obtained with an enhanced chemiluminescence detection kit (Amersham) and densitometric analysis was performed with Quantity One software (Bio-Rad, Hercules, CA, USA) normalizing with α-tubulin (whole cell extracts and cytosolic fractions) or lamin B1 (nuclear fractions).

Confocal microscopy analysis of JunD

Cells seeded on glass slides were fixed with 4% paraformaldehyde. A primary antibody against JunD (Pfarr *et al*., 1994) was used. The secondary anti-rabbit Alexa Fluor 546 antibody was from Invitrogen. In experiments aimed at demonstrating anti-JunD antibody specificity, a secondary anti-rabbit Alexa Fluor 488 antibody (Invitrogen) was used together with a fluor-

escent-labelled siRNA (siGLO RISC-free siRNA, Dharmacon, Lafayette, CO, USA) as transfection marker. Cell nuclei were stained with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad).

Activator protein-1 transcriptional activity
Cells were transfected with plasmids including a minimal region of the c-Jun promoter, which contains a wild-type or a mutated AP-1 site, linked to the reporter gene luciferase (c-Jun-luc and ΔAP-1/c-Jun-luc, respectively) constructed from the c-Jun-CAT plasmid. Cells were co-transfected with a renilla control reporter vector (Promega, Madison, WI, USA) to normalize experimental variations. Lipofectamine 2000 (Invitrogen) was used as transfection reagent. Luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega) and determined with a Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Co-transfections with c-Jun/c-Fos, JunD/c-Fos or c-Jun/Fra-1 chimaeric plasmids (Bakiri *et al*., 2002) were performed as positive controls of AP-1 transcriptional activation.

RNA interference
The double-stranded RNA duplexes were: three different human JunD siRNAs (5′-GGCCCUGGAGGAUUUAC ACtt-3′, 5′-GUAAAGUCUCGUUACGCCAtt-3′ and 5′-GC AUGAUGAAGAAGGACGCtt-3′) and three corresponding to human c-Jun (5′-GGAAGCUGGAGAGAAUCGCtt-3′, 5′-CCUAACAUUCGAUCUCAUUtt-3′ and 5′-CGUUAACA GUGGGUGCCAAtt-3′) from Ambion; human Tes and p27 siRNAs (ON-TARGETplus SMART pools) from Dharmacon; human p8 siRNA (5′-GGAGGACCCAGGACAGGAU-3′) and the non-targeted control siRNA (5′-UUCUCCGAAC GUGUCACGU-3′) from Eurogentec (Liege, Belgium). DharmaFECT 1 (Dharmacon) was used as transfection reagent. At 24 h after transfection, cells were trypsinized and seeded for further experiments.

Measurement of intracellular reactive oxygen species
After cannabinoid challenge, cells were incubated for 30 min with CM-H_2DCFDA (Molecular Probes), harvested and subjected to FACScan analysis as described in Gerald *et al*. (2004). Thirty thousand cells per analysis were recorded.

Statistical analysis
ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test was routinely used. Unless otherwise stated, results are expressed as mean ± s.d.

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References

Bakiri L, Matsuo K, Wisniewska M, Wagner EF, Yaniv M. (2002). Promoter specificity and biological activity of tethered AP-1 dimers. *Mol Cell Biol* **22**: 4952–4964.

Berdyshev EV, Schmid PC, Krebsbach RJ, Hillard CJ, Huang C, Chen N *et al*. (2001). Cannabinoid-receptor-independent cell signalling by N-acylethanolamines. *Biochem J* **360**: 67–75.

Blazquez C, Carracedo A, Barrado L, Real PJ, Fernandez-Luna JL, Velasco G *et al*. (2006). Cannabinoid receptors as novel targets for the treatment of melanoma. *FASEB J* **20**: 2633–2635.

Caffarel MM, Sarrio D, Palacios J, Guzman M, Sanchez C. (2006). Delta^9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. *Cancer Res* **66**: 6615–6621.

Carracedo A, Gironella M, Lorente M, Garcia S, Guzman M, Velasco G *et al*. (2006a). Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. *Cancer Res* **66**: 6748–6755.

Carracedo A, Lorente M, Egia A, Blazquez C, Garcia S, Giroux V *et al*. (2006b). The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. *Cancer Cell* **9**: 301–312.

De Petrocellis L, Melck D, Palmisano A, Bisogno T, Laezza C, Bifulco M *et al*. (1998). The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc Natl Acad Sci USA* **95**: 8375–8380.

Drusco A, Zanesi N, Roldo C, Trapasso F, Farber JL, Fong LY *et al*. (2005). Knockout mice reveal a tumor suppressor function for Testin. *Proc Natl Acad Sci USA* **102**: 10947–10951.

Eferl R, Wagner EF. (2003). AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* **3**: 859–868.

Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D *et al*. (2004). JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell* **118**: 781–794.

Giuliano M, Calvaruso G, Pellerito O, Portanova P, Carlisi D, Vento R *et al*. (2006). Anandamide-induced apoptosis in Chang liver cells involves ceramide and JNK/AP-1 pathway. *Int J Mol Med* **17**: 811–819.

Guzman M. (2003). Cannabinoids: potential anticancer agents. *Nat Rev Cancer* **3**: 745–755.

Guzman M, Duarte MJ, Blazquez C, Ravina J, Rosa MC, Galve-Roperh I *et al*. (2006). A pilot clinical study of Delta^9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. *Br J Cancer* **95**: 197–203.

Hampson AJ, Grimaldi M, Axelrod J, Wink D. (1998). Cannabidiol and (−)Delta^9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci USA* **95**: 8268–8273.

Hampson AJ, Grimaldi M, Lolic M, Wink D, Rosenthal R, Axelrod J. (2000). Neuroprotective antioxidants from marijuana. *Ann NY Acad Sci* **899**: 274–282.

Iuvone T, Esposito G, Esposito R, Santamaria R, Di Rosa M, Izzo AA. (2004). Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on beta-amyloid-induced toxicity in PC12 cells. *J Neurochem* **89**: 134–141.

Lallemand D, Spyrou G, Yaniv M, Pfarr CM. (1997). Variations in Jun and Fos protein expression and AP-1 activity in cycling, resting and stimulated fibroblasts. *Oncogene* **14**: 819–830.

Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L *et al*. (2006). Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J Pharmacol Exp Ther* **318**: 1375–1387.

Maccarrone M, Di Rienzo M, Battista N, Gasperi V, Guerrieri P, Rossi A *et al*. (2003). The endocannabinoid system in human keratinocytes. Evidence that anandamide inhibits epidermal differentiation through CB1 receptor-dependent inhibition of protein kinase C, activation protein-1, and transglutaminase. *J Biol Chem* **278**: 33896–33903.

Malumbres M, Barbacid M. (2001). To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* **1**: 222–231.

Mechta F, Lallemand D, Pfarr CM, Yaniv M. (1997). Transformation by ras modifies AP1 composition and activity. *Oncogene* **14**: 837–847.

Mechta-Grigoriou F, Gerald D, Yaniv M. (2001). The mammalian Jun proteins: redundancy and specificity. *Oncogene* **20**: 2378–2389.

Pfarr CM, Mechta F, Spyrou G, Lallemand D, Carillo S, Yaniv M. (1994). Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. *Cell* **76**: 747–760.

Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. (2006). Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *J Biol Chem* **281**: 14841–14851.

Sarnataro D, Pisanti S, Santoro A, Gazzerro P, Malfitano AM, Laezza C *et al*. (2006). The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits human breast cancer cell proliferation through a lipid raft-mediated mechanism. *Mol Pharmacol* **70**: 1298–1306.

Stickeler E, Kittrell F, Medina D, Berget SM. (1999). Stage-specific changes in SR splicing factors and alternative splicing in mammary tumorigenesis. *Oncogene* **18**: 3574–3582.

van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P *et al*. (1993). Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO J* **12**: 479–487.

Weitzman JB, Fiette L, Matsuo K, Yaniv M. (2000). JunD protects cells from p53-dependent senescence and apoptosis. *Mol Cell* **6**: 1109–1119.

Zhao Q, He Z, Chen N, Cho YY, Zhu F, Lu C *et al*. (2005). 2-Arachidonoylglycerol stimulates activator protein-1-dependent transcriptional activity and enhances epidermal growth factor-induced cell transformation in JB6 P+ cells. *J Biol Chem* **280**: 26735–26742.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).